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(54) **Recombinant fowlpox vaccine for protection against Marek's disease.**

(57) A recombinant fowlpox virus is disclosed which is useful as a vaccine for protection against Marek's Disease. The recombinant virus preferably contains a gene for one or more Marek's Disease Virus antigens such as glycoprotein B homologue, glycoprotein C homologue, glycoprotein D homologue, glycoprotein H homologue and tegument proteins, under the control of a poxvirus promoter within a region of the DNA of fowlpox virus which is not essential for virus growth.

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Field of the Invention

The present invention relates to a vaccine that protects against Marek's disease.

Description of Related Art

Marek's disease (MD) is a highly contagious neoplastic disease of domestic chicken that affects chickens worldwide and causes high mortality and condemnation if the chickens are not vaccinated at one day of age. MD is caused by a highly cell-associated oncogenic herpesvirus known as Marek's disease virus (MDV).

A number of live virus cell-associated vaccines are available that protect chickens against MD. These vaccines are maintained and administered in delicate cell-associated form. The vaccines need special handling and must be stored and transported in a frozen state in liquid nitrogen in order to maintain their viability and efficacy. These existing vaccines must be maintained and administered in cell-associated form, a condition that is costly and cumbersome.

The known vaccines contain the entire MDV genome, including sequences related to induction of pathogenesis. Although the existing vaccines against MD are either attenuated or are naturally apathogenic, viral mutation is known to occur in herpesviruses and there is a possibility that virulent pathogenic mutants may emerge in such vaccines. Such mutants could be less effective and even harmful.

Churchill et al, Nature, 221:744-747 (1969) and Okazaki et al, Avian Dis., 14:413-429 (1970) developed the first effective and safe vaccines against MD. These vaccines have been in use for the last 20 years and have reduced losses to the poultry industry worldwide. Other candidate vaccines based on serotype 2 naturally apathogenic MDV, Schat et al, J. Natl. Cancer Inst., 60, 1075-1082 (1978), or newly attenuated serotype 1 MDV, Rispens et al, Avian Dis., 16:108-125 (1972), and combinations of these viruses as bivalent vaccines, Witter, Avian Dis., 31:252-257 (1987), have helped provide a better protection against MD. All these vaccines, except the herpesvirus of turkeys (HVT) vaccine, require the storage and transportation in frozen state in liquid nitrogen and have to be administered as infected cells which calls for careful procedures to prevent inactivation of the vaccine. Even in the case of HVT vaccine, cell-associated viruses have been most widely used because they are more effective than cell-free virus in the presence of maternal antibodies, Witter et al, Avian Pathol., 8:145-156 (1978).

Recombinant DNA technology has allowed the construction of recombinant vaccines that contain only those desired viral genes or gene products that induce immunity without exposing the animal to genes that may induce pathological disorders. Pox viruses, including avipox virus, especially the fowlpox virus (FPV), provide excellent models for such vaccines. These viruses have a large DNA molecule with numerous nonessential regions that allow the insertion of several immunogenic genes into the same virus for the purpose of creating multivalent vaccines. These multivalent vaccines may induce cell-mediated as well as antibody-mediated immune response in a vaccinated host. Vaccinia virus (VV) has been used extensively for this purpose and a number of VV recombinants have been constructed that express a variety of foreign genes including those that elicit neutralizing antibodies against glycoproteins of herpes simplex virus (HSV) type 1, Blacklaws et al, Virology, 177:727-736 (1990). Similarly, there are a number of reports describing the expression of foreign genes by recombinant FPV, Boyle et al, Virus Res., 10:343-356 (1988) and Ogawa et al, Vaccine, 8:486-490 (1990).

MDV homologues of the HSV gene coding for glycoproteins B, C, D, H, and I (gBh, gCh, gDh, gHh and gIh) have recently been cloned and sequenced, Coussens et al, J. Virol., 62:2373-2379 (1988), Ross et al, J. Gen. Virol., 70:1789-1804 (1989), Ross et al, J. Gen. Virol., 72:939-947 (1991), Ross et al, European Patent Application International Publication No. WO 90/02803 (1990).

It is an object of the present invention to provide a novel, effective, and safe vaccine against MD that exposes and immunizes the chicken only to the immunogenic gene product(s) of the MDV without exposure to its pathogenic gene products. The novel vaccine of the present invention, that lacks sequence related to pathogenic elements of MDV, is available in cell-free form and induces effective immunity against virulent MD. This is far more desirable than the existing vaccines.

It is also an object of the invention to provide cell-free vaccine against MD containing recombinant (rec) FPV that can be lyophilized, stored, and used under normal conditions thereby obviating costly and laborious procedures of storing the vaccine in liquid nitrogen, delicate handling, and administering which are necessary with existing cell-associated MD vaccines. For example, the vaccine of the present invention, after lyophilization, can be stored, handled, and transported at ambient temperature (20-22°C) and stored at 4°C for prolonged periods of time. The vaccine can also be stored in a frozen state wherein the cell-free recombinant virus is present in an aqueous solution which is frozen and stored at, for example, -20°C or -70°C.

This invention relates to the development of a novel recombinant FPV vaccine that contains a gene which encodes a glycoprotein B homologue (gBh) of MDV, expresses this gBh gene in cell culture and provides a

strong protection against MD in the natural host (chicken), when administered as a cell-free material. In addition, the vaccine will also protect against fowlpox.

A further object of the invention is to provide recombinant FPV vaccines against MD in which gBh gene of MDV as well as other MDV genes such as those coding for glycoprotein C homologue, glycoprotein D homologue, tegument proteins and glycoproteins from different serotypes of MDV are inserted into FPV for the purpose of creating a broad-spectrum vaccine effective against several isolates of MDV.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the construction of insertion vector pNZ1729R;
FIG. 2 shows the sequences of 10 oligonucleotides (SEQ. ID. NOS. 1-10) used for construction of pNZ1729R insertion vector;
FIG. 3 shows the steps taken to clone MDV gBh of HSV gene;
FIG. 4 shows the construction of transfer vector pNZ29RMDgB-S; and
FIG. 5 shows the immunoprecipitation of cells infected with rec.FPV/MDVgBh or with GA strain of MDV.

The development of the recombinant FPV expressing the antigen gene of MDV and protecting the chickens against MD involved a multi-step procedure including: 1) construction of an insertion vector using a non-essential region of FPV DNA cloned into a vector; 2) cloning and sequencing of the MDV antigen gene; 3) construction of a transfer vector including the antigen gene and the marker gene in opposite directions and under the control of different poxvirus promoters; 4) transfection of FPV infected host cell cultures with this transfer vector, generation of recombinants and purification of recombinants expressing marker gene; 5) demonstration of expression of the MDV antigen in host cell cultures infected with the recombinant FPV; and 6) demonstration of full protection offered by FPV recombinant vaccine against death and tumors caused by virulent tumorigenic MDV.

Any virus is usable as FPV in the present invention as far as it is classified into the genus FPV in a broad sense but preferred are those capable of growing in cells of fowls such as chicken, turkey, duck, etc. Specific examples include FPV in a narrow sense such as ATCC VR-251, ATCC VR-250, ATCC VR-229, ATCC VR-249, ATCC VR-288, Nishigawara strain, Shisui strain, CEVA strain, etc.; and those akin to FPV in a narrow sense and used to fowl live vaccine strain such as NP strain (chick embryo habituated pigeonpoxvirus Nakano strain), etc. These strains are all commercially available and easily accessible.

Any DNA region is usable as non-essential region in the present invention as far as it is nonessential to proliferation of FPV. Specific examples of the non-essential region include regions which cause homologous recombination with EcoRI fragment (about 7.3kbp), Hind III fragment (about 5.2kbp), EcoRI-Hind III fragment (about 5.0kbp), BamHI fragment (about 4.0kbp), etc. of the DNA of NP strain and the like.

Any vector is usable as vector in construction of the insertion vector for use as the vehicle to transfer the antigen gene of MDV to FPV. Specific examples of the vector include a plasmid such as pBR322, pBR325, pUC7, pUC8, pUC18, etc.; a phage such as λ phage, M13 phage, etc.; a cosmid such as pHC79, etc.

Any antigen gene of MDV is usable as antigen gene in the present invention as far as it is able to induce protection against Marek's disease. Specific examples of the antigen gene of MDV include gene coding for gBh, gene coding for gCh, gene coding for gDh, gene coding for gHf, gene coding for glh, tegument gene, etc.; and variants of them. Preferable antigen gene is the gene coding for gBh, because FPV containing the gene coding for gBh protects the host from very virulent strains of MDV, such as Md5 strain, very well.

Any marker gene is usable as marker gene in the present invention as far as it is able to expression in host cells. Specific examples of the marker gene include lacZ gene of E. coli., Ecogpt gene, etc.

Any host cell is usable as FPV host cell in the present invention as far as FPV can grow there. Specific examples are chicken-derived culture cells such as chick embryo fibroblast, etc. Furthermore, chick chorio-allantoic membrane is also naturally included in the category of host cell.

Any promoter is usable as promoter for antigen gene in the present invention as far as it functions in the host cell. Specific-examples of the promoter include promoter of vaccinia virus gene coding for 7.5 K polypeptide, promoter of vaccinia virus gene coding for 11 K polypeptide, promoter of vaccinia virus gene coding for thymidine kinase polypeptide, etc.; synthetic poxvirus promoter including early promoter and late promoter, etc.; and variants thereof, etc. Preferable promoters are the synthetic poxvirus promoter including early promoter and late promoter (A. J. Davidson et al., J. Mol. Biol., 215, 749-769 & 771-784 (1989)) and those variants (For Example, the base sequence of the synthetic poxvirus promoter is TTTTTTTTTTTTTGGCATATAAATAATAAA TACAATAATTAATTACGCGTAAAAATTGAAAAACTATTCTAATTTATTGCACTC).

One of the example of the insertion vector for use as the vehicle to transfer the gBh gene of MDV to FPV is pNZ1729R. This insertion vector was derived through multiple molecular manipulation of a cloned nonessential region of FPV DNA, Yanagida et al, European Patent Application Publication No. 0 284 416 (1988), and

insertion of a lacZ bacterial gene as a reporter gene and creation of a multiple cloning site for insertion of foreign genes into this region of FPV DNA. A 3.0 kilobase (kb) pair fragment of FPV DNA was cloned into an appropriate cloning site of the bacterial plasmid pUC18. The resulting construct was altered by several restriction endonuclease (RE) digestions, religation and insertion of a multiple cloning site. The beta-galactosidase gene (lacZ) of *E. coli* was inserted into a unique RE site of this FPV DNA after having been linked to a poxvirus promoter followed by an initiation ATG codon and terminated with a transcriptional termination signal for poxvirus early promoter, Yuen et al, PNAS USA, 84:6417-6421 (1987). When this construct was transfected into FPV infected cells, recombinant viruses were generated that produced the product of the lacZ gene; the betagalactosidase which in turn gave rise to blue plaques in the presence of the Blu-o-gal substrate.

In separate experiments the MDV gBh gene homologue of HSV gB gene was cloned into the bacterial plasmid pUC18. The nucleotide sequence of this gene was determined by analyzing a set of deletion mutants by the dideoxy chain termination reaction, Sanger et al, PNAS DSA, 74:5463-5467 (1977). One of these mutants (pUCgBdBl3), which was found to contain the entire coding region of MDVgBh, was used for construction of the transfer vector pNZ29RMDgB-S. Site specific mutagenesis, Tsurushita et al, Gene, 62:135-139 (1988) was used to change a potential poxvirus early transcription termination signal, Yuen et al, PNAS USA, 84:6417-6421 (1987), in the gBh gene of MDV without changing the amino acid of the translation product. In addition, a number of molecular procedures including RE digestion, ligation, site specific mutation, polymerase chain reaction (PCR) with appropriate primers were applied to properly insert the gBh gene of MDV from the mutant pUCgBdBl3 into the pNZ1729R insertion vector to create the transfer vector, pNZ29RMDgB-S.

Purified pNZ29RMDgB-S plasmid was used to transfect CEF cultures infected with a large-plaque phenotype isolated from a vaccine FPV (CEVA strain) and the progeny virus released by these cells were assayed for recombinant virus producing blue plaques in the presence of Blu-o-gal. Recombinants were purified and tested for stability, structure of viral DNA, expression of lacZ and synthesis of gBh antigen of MDV in cell culture. Purified recombinants produced betagalactosidase (blue plaques) and the gBh antigen as tested by immunofluorescence (IF) or immunoprecipitation assays using monoclonal antibody specific to MDV gBh antigen or convalescent serum from an MDV infected chicken. Three identical bands of 100 kd, 60 kd, and 49 kd in molecular weight were observed in extracts of cells infected with rec.FPV/MDVgBh and MDV. These polypeptides were also shown to be glycosylated. Similar glycoproteins were identified with the same monoclonal antibody in the MDV "B antigen complex" and were referred to as gP100, gP60, and gP49, Sithole et al, J. Virol., 62:4270-4279 (1988). Our finding is the first clear demonstration that MDV gBh gene codes for these three glycoproteins referred to as the "B antigen complex".

Three-week-old chickens were vaccinated with the recombinant FPV expressing the MDV gBh antigen and sera from these chickens were assayed for the presence of antibodies against MDV infected cells in culture. Positive antibodies to MDV gBh antigen were found in these sera indicating that the MDV gBh gene was efficiently expressed in the chicken and induced an immune response.

Separate groups of unvaccinated chickens were vaccinated at one day of age with parental FPV, recombinant FPV (rec.FPV) expressing MDV gBh antigen or a conventional MD vaccine (HVT). All groups were later challenged with tumorigenic GA isolate of MDV. Chickens vaccinated with rec.FPV as well as those vaccinated with HVT were fully protected against MD whereas the unvaccinated control chickens and those vaccinated with parental FPV died or had MD specific tumors.

Similar vaccination trials were performed to determine the effect of vaccine dose, route of vaccination, and promoter strength on immunity against MD and the ability of rec.FPV/MDVgBh to protect against very virulent strains of MDV. Chickens vaccinated with a dose of 10^4 PFU of rec.FPV/MDVgBh were protected against challenge with three different strains of MDV tested. Vaccination route; intramuscular (IM) intraabdominal (IA) or vaccination by IM and IA did not seem to alter the level of protection as all chickens from each group were fully protected against MD. We generated another rec.FPV (rec.FPV/MDVgBh-P7.5) which expresses the MDVgBh gene under the control of vaccinia virus 7.5 kd protein gene promoter (P7.5), Ventakesan et al, Cell, 125:805-813 (1981) and tested its ability to protect against MD in comparison with the rec.FPV/MDVgBh which is driven by a poxvirus synthetic promoter (Ps). The rec.FPV/MDVgBh-P7.5 also gave a good protective immunity against MD but not as good as that obtained by vaccination with rec.FPV/MDVgBh driven by the poxvirus synthetic promoter. We also showed the ability of rec.FPV/MDVgBh to protect against two very virulent strains of MDV (RBIB; Schat et al, Avian Pathol., 11:593-605 (1982) and Md5; Witter et al, Avian Dis., 24:210-232 (1980)).

The cell-free vaccine of the present invention can be prepared by a variety of techniques. For example, a cell culture in which the recombinant virus of the present invention can grow and replicate is infected with the recombinant virus of the present invention. The cell culture is then incubated until the virus has had an opportunity to replicate in the cell culture. The cells are then harvested and disrupted. The cell debris can then be centrifuged to produce a pellet of cell debris at the bottom of the centrifuge tube and a substantially higher-titer cell-free supernatant containing the recombinant virus. The cell-free supernatant, which will consist primarily

of the cell culture medium and the recombinant FPV, is then used as a vaccine containing the recombinant virus. In the alternative, the cell-free supernatant is lyophilized to produce a lyophilized vaccine which is reconstituted with a pharmaceutically acceptable carrier such as physiological saline prior to use.

The vaccine of the present invention can be administered to chickens in any manner which allows the recombinant virus in the vaccine to infect the chickens and produce a protective immune response. For example, the vaccine can be applied to the chickens subcutaneously (s.c.) by scratching the skin or injection with a needle or other implement which contains the virus. The recombinant virus can also be dissolved or suspended in the drinking water of chickens for oral administration. The virus may also be mixed with a solid carrier (e.g. chicken feed) for oral administration. Other modes of administration are also contemplated such as inhalation by use of an aerosol or spray, intravenous administration, intramuscular administration, intraperitoneal administration, wing web administration, etc.

A preferred dose for injection appears to be 10^4 plaque forming units (PFU) per chicken in 0.1 ml of a physiologically acceptable liquid carrier. Thus, the injectable solution will contain 10^5 PFU/ml of carrier, usually between 10^4 to 10^6 PFU/ml of carrier. The dose and route of administration should be selected to elicit a protective immune response.

The recombinant virus of the present invention can contain a gene encoding more than one antigen such as one or more antigens selected from the group consisting of glycoprotein B homologue, glycoprotein C homologue, glycoprotein D homologue, glycoprotein H homologue and tegument proteins. In the alternative, multiple recombinant viruses can be included in the vaccine wherein each individual virus expresses a single gene. It is believed that by exposing the chickens to multiple antigens of the Marek's Disease Virus which elicit a protective immune response, improved protection may be achieved.

In addition to the specific glycoproteins mentioned above, it is also contemplated in accordance with the present invention that fragments of the genes coding for the above-mentioned antigens or variants of the genes which code for variants of the above-mentioned antigens may also be useful as long as the resulting protein (antigen) elicits a protective immune response. It is contemplated that such fragments or variants would code for proteins (antigens) which have substantially the same amino acid sequence as the natural proteins to thereby elicit a substantially equivalent immune response in the host. The fragments or variants will usually encode a protein which has more than 80%, preferably more than 90%, and more preferably more than 95% homology to the natural protein.

The recombinant virus of the present invention has the gene for the antigen inserted into the virus under control of appropriate promoters, terminators, etc. so that the virus, after it infects a host cell, can express the protein (antigen) thereby eliciting an immune response in the host. Ps, which is a strong synthetic poxvirus promoter which produces high levels of expression during both the early and late stages of infection, is particularly useful. Promoter P7.5 is also useful. Other poxvirus promoters may also be used.

EXAMPLE 1

Construction of insertion vector pNZ1729R (Fig. 1)

A 3.0 kb HpaI-SpeI fragment from a 7.3 kb EcoRI fragment of FPV NP strain, Yanagida et al, European Patent Application No. 0 284 416 (1988), was subcloned into pUC18 in several steps in a conventional manner. After eliminating all multiple cloning sites from both junction regions between pUC18 and FPV DNA, a multiple cloning site (HindIII-EcoRI 52 bp from pUC18) was inserted into two adjacent EcoRV sites in the cloned FPV fragment with linkers (HindIII linker; 5'-CAAGCTTG-3', EcoRI linker; 5'-GGAATTCC-3') to make pNZ133SR.

A 3.5 kb EcoRI-HindIII fragment (shown in Fig. 1 right center) was derived by ligating-annealing oligos 1 (SEQ. ID. NO. 1) and 2 (SEQ. ID. NO. 2) (Fig. 2; containing a fowlpox promoter followed by an ATG codon for lacZ), to lacZ gene (from pMC1871 and pMA001), Shirakawa et al, Gene, 28:127-132 (1984) and annealing oligos 3 (SEQ. ID. NO. 3), 4 (SEQ. ID. NO. 4), 5 (SEQ. ID. NO. 5), 6 (SEQ. ID. NO. 6), 7 (SEQ. ID. NO. 7) ,8 (SEQ. ID. NO. 8), 9 (SEQ. ID. NO. 9) and 10 (SEQ. ID. NO. 10) (Fig. 2; containing synthetic poxvirus promoter, followed by a multiple cloning site and a two directional poxvirus early transcriptional termination signal (SEQ. ID. NO. 11), Yuen et al, PNAS, 88:6417-6421 (1989)). The 3.5 kb EcoRI-HindIII fragment was inserted in pNZ173SR to make the pNZ1729R insertion vector.

EXAMPLE 2

Cloning of MDV gBh gene (Fig. 3)

The MDV gBh of HSV from a BamHI I3 (5.2 kb) and K3 (3.6 kb) fragment of MDV GA strain was cloned

into pUC18 plasmid. A 2.8 kb BamHI-SalI subfragment from I3 fragment and a 1.1 kb BamHI-EcoRI subfragment from K3 fragment were ligated with EcoRI, SalI digested pUC18.

The overall sequence of the putative MDV gBh was determined by sequencing a set of deletion mutants by the Sanger dideoxy chain termination method, Sanger et al, PNAS USA, 74:5463-5467 (1977). The nucleotide and amino acid sequences (SEQ. ID. NOS. 12 and 13) were found to be identical with the published sequences of the gBh of RBIB strain of MDV, Ross et al, J. Gen. Virol., 70:17B9-1804 (1988).

EXAMPLE 3

Construction of transfer vector DNZ29RMDgB-S (Fig. 4)

One of the deletion mutants for sequencing the MDV gBh gene, named pUCgBdBI3, that contained the entire coding region of the gBh with about 250 bp 5' flanking region was chosen for insertion into insertion vector pNZ1729R.

The plasmid pLELR, which was derived from pNZ1037, Ogawa et al, Vaccine, 8:488-490 (1990), with synthetic adapter

5'-CGAATTCGTCGAC-3' (SEQ. ID. NO. 14)

3'-TCGAGCTTAAGCAGCTGTTAA-5' (SEQ. ID. NO. 15)

to make a SalI site next to EcoRI site, was digested with SmaI and EcoRI and was ligated with a 1.9 kb HindIII (Klenow-blunt)-BamHI fragment and a 1.1 kb BamdIII-EcoRI fragment, both from pUCgB-dB13. Site specific mutagenesis was used to eliminate about 250 bp 5'flanking region and to change a potential poxvirus early transcription termination signal in the gBh gene of pUCgB7.5 (TTTTTTT; nucleotides 382-388 in SEQ. ID. NO. 12) to TATTTTT. Oligonucleotides for site specific mutagenesis of(P7.5-gB) 34mer; was oligonucleotide (SEQ. ID. NO. 16) for site-specific mutagenesis of (TTTTTTT) 26mer; was (SEQ. ID. NO. 17).

In order to create a new BamHI site in front of translation initiation codon (ATG) of gBh for connecting the gBh gene with a synthetic promoter, PCR was performed with synthetic oligonucleotides (SEQ. ID. NO. 18) and (SEQ. ID. NO. 19).

About 200 bp BamHI-XbaI fragment from the PCR product was ligated with a 2.7kb XbaI-SalI fragment of gBh and BamHI, SalI digested vector pNZ1729R to make transfer vector pNZ29RMDgB-S.

EXAMPLE 4

Generation and purification of recombinant FPV/MDVgBh

CEF cultures propagated as monolayers were infected with 0.1 multiplicity of infection (moi) of a large-plaque phenotype virus isolated from a vaccine preparation of FPV. Three hours after infection, cells were dispersed by trypsinization and brought into suspension. $2x10^7$ cells from this suspension were mixed with 10 micrograms (μg) of transfer vector pNZ29RMDgB-S in a Cell Porator (Bethesda Research Laboratories, Inc., Bethesda, MD) according to the manufacturer's specifications. The mixture of cell suspension and the transfer vector DNA in 0.8 ml of Saline G containing 0.14M NaCl, 0.5 mM KCl, 1.1 mM $NaIHPO_4$-12 H_2O, 1.5 mM KH_2PO_4, 0.5 mM $MgCl_2.6H_2O$, and 0.011% glucose was subjected to electroporation under an electric field of 300 V x cm^{-1} at room temperature using 330 μF of capacitance. Transfected cells were then incubated at 37°C for 72 hours (h) and were then lysed by three cycles of freezing and thawing. The released virus was screened for recombinants as follows.

Secondary CEF cultures were infected with serial ten-fold dilutions of the progeny virus from lysates and overlayed with 10 ml of agar solution containing growth medium and allowed to harden at room temperature and incubated at 37°C until typical FPV plaques appeared. Another agar overlay containing 250 μg/ml of Blu-o-gal (BRL) was added to each plate and incubated at 37°C for another 48 h. Blue plaques appeared at a rate of approximately 1% of the total progeny virus. These blue plaques were removed from agar and the recombinant virus released from this agar was further purified in the same manner until all FPV plaques produced blue plaques when assayed in the presence of Blu-o-gal. This process usually took only three passages. The purified recombinant virus was named rec.FPV/MDVgBh. The DNA from this rec.FPV/MDVgBh was analyzed by Southern blot hybridization and found to contain the MDVgBh and lacZ genes at the expected positions. The virus rec.FPV/MDVgBh was deposited at the American Type Culture Collection (12301 Parklawn Drive, Rockville, Maryland, U.S.A.) on June 20, 1991 and was assigned deposit number ATCC-VR-2330 under the

conditions of the Budapest Treaty.

EXAMPLE 5

Expression of MDV gBh antigen in cell culture

In order to show that rec.FPV/MDVgBh synthesizes the gBh antigen, CEF cultures infected with this virus were examined by IF using antibodies specifically raised against this antigen. CEF cultures infected with rec.FPV/MDVgBh were incubated at 37°C until typical FPV plaques were developed. These cultures were fixed in cold acetone, then reacted with appropriate dilutions of convalescent chicken serum against the GA strain of MDV or a monoclonal antibody specific to MDV gB antigen, Silva et al, Virology, 136:307-320 (1984). These cultures were then reacted with fluorescein conjugated anti-chicken or anti-mouse immunoglobulins, respectively, and after thorough washing to remove non-specific staining they were examined with a microscope under ultraviolet (UV) illumination. CEF cultures infected with non-recombinant parental FPV were similarly stained. Specific cytoplasmic staining of cells was observed in cultures infected with the rec.FPV/MDVgBh and not in cultures infected with the non-recombinant parental FPV. These observations clearly showed that the recombinant virus was capable of synthesizing the product of gBh gene of MDV in cell cultures.

Western blot analysis of proteins from recombinant FPV-infected cells did not reveal the expected glycoprotein bands associated with gBh gene when lysates were boiled in buffer as in normal conditions of the assay. However, when solubilized with sample buffer at room temperature instead of 100°C a high molecular weight band was detected with a Rf value similar to that in MDV infected cell lysates solubilized at room temperature. In order to clearly show the three species of glycoproteins previously shown to be associated with MDV "B antigen complex", we examined the expression of the gBh gene by immunoprecipitation as described by Silva et al, Virology, 136:307-320 (1984). Secondary CEF cultures infected with either parental or recombinant FPV at an moi of 15 were incubated at 37°C for 4 hours. Then, the medium was replaced with 1 ml of fresh Methionine-free medium and incubated for another hour. About 40 uCi of ^{35}S-Methionine (NEN, Wilmington, DE) was then added and the cultures were incubated for an additional 12 hours. Cells were washed twice in PBS, scraped, and transferred to a 15 ml Falcon tube. Cells were centrifuged, resuspended in lysis buffer (150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, and 10 mM Tris HCl, pH 7.5) and incubated at room temperature for 30 minutes. One half volume of 10% (v/v) *S. aureus* Cowan 1 (SAC) was added to cell lysate, and incubated for 30 minutes on ice. The lysate was then centrifuged and the supernatant was collected. About 3 µl of monoclonal antibody, IAN86 against MDV "B antigen complex", Silva et al, Virology, 136:307-320 (1984), was added to 100 µl of lysate and incubated for 30 minutes on ice. An equal volume of 10% (v/v) SAC was added and incubated on ice for 30 minutes. Immunoprecipitates were then washed, suspended in sample buffer, and then boiled. After centrifugation, supernatant was analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, Laemmli, Nature, 207:680-685 (1970). Figure 5 shows the result of immunoprecipitation with a monoclonal antibody (IAN86) specific to the MDV "B antigen complex". Lane 1 is a control containing non-recombinant fowlpox virus cell lysate. Three identical bands of 100 kd, 60 kd and 49 kd in molecular weight were observed in extracts of cells infected with rec.FPV/MDVgBh (Figure 5, lane 2) and MDV (Figure 5, lane 3). These glycoproteins were also shown to be glycosylated by demonstrating that they uptake radioactively labelled glucosamine. Similar glycoproteins were identified with the same monoclonal antibody in the MDV "B antigen complex" and were referred to as gP100, gP60, and gP49, Sithole et al, J. Virol., 62:4270-4279 (1988).

This is the first clear demonstration that MDV gBh gene codes for these three glycoproteins previously referred to as the "B antigen complex". The latter two glycoproteins are believed to be the cleavage products of gP100 which may explain the rather weak signals obtained for this glycoprotein in our immunoprecipitation of cell lysates from late stages of infection (Figure 5).

EXAMPLE 6

Ability of the rec.FPV/MDVgBh to induce humoral immunity against MDV gBh antigen in chickens

A group of five, 3-week-old specific pathogen free (SPF) line O chickens raised in this laboratory were injected with 10^6 infectious doses (PFUs) of rec.FPV/MDVgBh intramuscularly while another group of five similar chickens were injected with the non-recombinant FPV. Similar booster inoculations were given after 2 and 4 weeks. Sera were collected from all chickens two weeks after the last inoculation. Sera were tested for the presence of antibodies to MDV gBh antigen. Coverslip monolayer cultures of CEF were infected with MDV GA strain and incubated at 37°C until typical MDV plaques were visible with the light microscope. These cultures

were then reacted with appropriate dilutions of sera from chickens of both groups followed by extensive washing and reaction with fluorescein conjugated goat anti-chicken immunoglobulin. Cultures were then examined by a microscope equipped with an UV illuminator. Sera from chickens immunized with rec.FPV/MDVgBh reacted positively with MDV infected cells and stained cytoplasmic antigens typical of gBh antigen of MDV. Sera from chickens immunized with the non-recombinant FPV failed to stain the gBh antigen of MDV. These results demonstrated clearly that the rec.FPV/MDVgBh is capable of inducing specific antibodies against the gBh antigen of MDV when injected into chickens.

EXAMPLE 7

rec.FPV/MDVgBh fully protects chickens against challenge with virulent tumor inducing MDV

Separate groups of 1-day-old chicks from 15x7 chicken line susceptible to MD were vaccinated with 10^6 plaque forming units (PFU) of rec.FPV/MDVgBh, 10^6 PFU of parental FPV or 10^3 PFU of HVT vaccine. Another group of similar chicks was kept unvaccinated. All were kept in strict isolation. At 12 days of age all were challenged with $1x10^3$ PFU of virulent tumor causing GA strain of MDV. A fifth group of chickens were neither vaccinated nor challenged. Mortality caused by MD was recorded during the trial and at the end of the 10 week trial all chickens were examined for gross lesions and tumors typical of MD. The results of this study are presented in Table 1.

In a second trial one-day-old chicks were either vaccinated intraabdominally (IA) with 10^3 PFU of HVT or vaccinated with 10^4 PFU of rec.FPV/MDVgBh half intra-muscularly (IM) and half IA. One group received the vaccine only IA and another group received the vaccine IM. One group received rec.FPV/MDVgBh-P7.5 in which the MDVgBh gene is driven by the vaccinia virus 7.5 kd protein promoter (P7.5). At six days post vaccination, six groups were challenged with 10^3 PFU of pathogenic GA strain of MDV while three other groups each were challenged with 10^3 PFU of very virulent strains of MDV (RBIB strain, Schat et al, Avian Pathol., 11:593-605 (1982) or Md5, Witter et al, Avian Dis., 24:210-232 (1980)). The results of this study are presented in Table 2.

Table 1: Protection against MD by rec.FPV/MDVgBh.

Lot	Vaccine	Challenge	MD mortality	MD gross lesions	%MD
1	None	GA-MDV	9/15	1/6	66
2	HVT	GA-MDV	0/15	0/15	0
3	rec.FPV/MDVgBH	GA-MDV	0/15	0/15	0
4	parental FPV	GA-MDV	3/15	3/12	40
5	None	None	0/10	0/10	0

Table 2. Protection against different strains of MDV by rec.FPV/MDVgBh.

Lot.	Vaccine	Vaccine route	MDV challenge strain	MD mortality	MD gross lesions	%MD
1	None		GA	8/10	1/2	90
2	HVT	IA	GA	0/10	0/10	0
3	rec.FPV/MDVgBh	IA&IM	GA	0/10	0/10	0
4	rec.FPV/MDVgBh-P7.5	IA&IM	GA	1/10	1/9	20
5	rec.FPV/MDVgBh	IA	GA	0/10	0/10	0
6	rec.FPV/MDVgBh	IM	GA	0/10	0/10	0
7	None		Md5	8/10	2/2	100
8	rec.FPV/MDVgBh	IA&IM	Md5	0/10	0/10	0
9	HVT	IA	Md5	0/10	0/10	0
10	None		RB1B	9/10	0/1	90
11	rec.FPV/MDVgBh	IA*IM	RB1B	0/10	1/10	10
12	HVT	IA	RB1B	1/10	0/9	10
13	None		None	0/5	0/5	0

A significant number of unvaccinated chickens in groups challenged with all three strains died of MD or had MD specific tumors and lesions at the end of the trial. Those vaccinated with rec.FPV/MDVgBh or HVT were fully protected against the GA and the very virulent Md5 strains. Those vaccinated with either of the above

vaccines were also significantly and equally protected against the very virulent RBIB strains of MDV. There was no significant difference between the level of protection induced by vaccination route as all birds vaccinated IM, IA or IM&IA and challenged with the GA strain of MDV were fully protected against MD. The rec.FPV-/MDVgBh which expresses the MDVgBh gene under the control of a poxvirus synthetic promoter was superior to the rec.FPV/MDVgBh-P7.5 which expresses the same gene under the control of vaccinia virus P7.5 promoter in that it fully protected against MD while the latter recombinant did offer a significant protection but not as well as the recombinant driven by the poxvirus synthetic promoter.

A significant number of unvaccinated chickens and those vaccinated with parental FPV that were challenged with MDV died of MD or had MD lesions and tumors at the end of trial. Chickens vaccinated with rec.FPV-/MDVgBh were fully protected against MD with no mortality and no lesions typical of MD. Similarly, all chickens vaccinated with HVT were protected. No mortality or lesions were present in chickens that were not injected with MDV. These results showed that the rec.FPV/MDVgBh fully protected chickens against MD, just as well as the widely used commercial HVT vaccine.

EXAMPLE 8

Preparation of cell free vaccine from recombinant FPV/MDVgBh

Confluent monolayers of chicken embryo fibroblast cultures containing about $4x10^7$ cells in plastic tissue culture dishes are infected with 1 ml of rec.FPV/MDVgBh stock containing approximately $1x10^6$ PFU of the virus and allowed to incubate at 37°C for 2 hours. At this time, 20 ml of fresh culture medium is added to each plate. Cultures are then incubated in a 5% CO_2 incubator at 37°C for 3 to 4 days until the entire monolayer of cells shows signs of infection. At this time, cell monolayer is scraped off from the culture dish using a cell lifter (Costar Corp.). Cells are then pelleted by centrifugation and suspended in 5 ml of the original culture medium and sonicated at half strength on ice for 60 seconds using a Braun-Sonic U sonicator (Braun Co. Ltd.). Sonicated material is then centrifuged to remove cell debris and the supernatant fluid is added to the remainder of the original culture medium. This vaccine preparation is then dispensed in 1 ml aliquots, placed in glass vials and stored at -70°C in a freezer.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: NAZERIAN, Keyvan
LEE, Lucy F.
YANAGIDA, Noboru
OGAWA, Ryohei
LI, Yi

(ii) TITLE OF INVENTION: RECOMBINANT FOWLPOX VACCINE FOR PROTECTION AGAINST MAREK'S DISEASE

(iii) NUMBER OF SEQUENCES: 19

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: Patent In Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Murphy Jr., Gerald M.
(B) REGISTRATION NUMBER: 28,977
(C) REFERENCE/DOCKET NUMBER: 1644-103P

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 48 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AATTCGAGCT CGGATCGTTG AAAAAATAAT ATAGATCCTA AAATGGAA 48

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GATCTTCCAT TTTAGGATCT ATATTATTTT TTCAACGATC CGAGCTCG 48

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AGCTTTTTTT TTTTTTTTTT TTTGGCATAT AAATAATAAA TACAATAATT AATTA 55

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGCGTAATTA ATTATTGTAT TTATTATTTA TATGCCAAAA AAAAAAAAAA AAAAA 55

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGCGTAAAAA TTGAAAAACT ATTCTAATTT ATTGCACTCG 40

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GATCCGAGTG CAATAAATTA GAATAGTTTT TCAATTTTTA 40

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 42 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GATCCCCGGG CGAGCTCGCT AGCGGGCCCG CATGCGGTAC CG 42

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 42 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TCGACGGATC CGCATGCGGG CCCGCTAGCG AGCTCGCCCG GG 42

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TCGACCCGGT ACATTTTTAT AAAAATGTAC CCGGGGATC 39

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GATCCCCGGG TACATTTTTA TAAAAATGTA CCGGG 35

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

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ATTTTTATAA AAAT                                                    14
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(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 3209 base paIrs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 357..2951

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

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AATTAAATGT GGCGAATTGC ACATCTGTCG TGCCGACAGT TTGCAGATCA ACAGCAATGG   60

AGACTATGTA TGGAAAAATG GAATATATAT AACATATGAA ACCGAATATC CACTTATAAT  120

GATTCTGGGG TCAGAATCAA GCACTTCAGA AACGCAAAAT ATGACTGCAA TTATTGATAC  180

AGATTTTTTT CGTTGCTTTA TTCTATTTTG CAGTATATGG CCCCCGTTAC GGCAGATCAG  240

GTGCGAGTAG AACAGATTAC CAACAGCCAC GCCCCCATCT GACCCGTCCA ATATTCTTGT  300

GTCCCTGCAT TTTATCTCAC ACAATTTATG AACAGCATCA TTAAGATCAT CTCACT      356

ATG CAC TAT TTT AGG CGG AAT TGC ATT TTT TTC CTT ATA GTT ATT CTA    404
Met His Tyr Phe Arg Arg Asn Cys Ile Phe Phe Leu Ile Val Ile Leu
  1               5                  10                  15

TAT GGT ACG AAC TCA TCT CCG AGT ACC CAA AAT GTG ACA TCA AGA GAA    452
Tyr Gly Thr Asn Ser Ser Pro Ser Thr Gln Asn Val Thr Ser Arg Glu
             20                  25                  30

GTT GTT TCG AGC GTC CAG TTG TCT GAG GAA GAG TCT ACG TTT TAT CTT    500
Val Val Ser Ser Val Gln Leu Ser Glu Glu Glu Ser Thr Phe Tyr Leu
         35                  40                  45

TGT CCC CCA CCA GTG GGT TCA ACC GTG ATC CGT CTA GAA CCG CCG CGA    548
Cys Pro Pro Pro Val Gly Ser Thr Val Ile Arg Leu Glu Pro Pro Arg
     50                  55                  60

AAA TGT CCC GAA CCT AGA AAA GCC ACC GAG TGG GGT GAA GGA ATC GCG    596
Lys Cys Pro Glu Pro Arg Lys Ala Thr Glu Trp Gly Glu Gly Ile Ala
 65                  70                  75                  80
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ATA TTA TTT AAA GAG AAT ATC AGT CCA TAT AAA TTT AAA GTG ACG CTT    644
Ile Leu Phe Lys Glu Asn Ile Ser Pro Tyr Lys Phe Lys Val Thr Leu
                85                  90                  95

TAT TAT AAA AAT ATC ATT CAG ACG ACG ACA TGG ACG GGG ACG ACA TAT    692
Tyr Tyr Lys Asn Ile Ile Gln Thr Thr Thr Trp Thr Gly Thr Thr Tyr
            100                 105                 110

AGA CAG ATC ACT AAT CGA TAT ACA GAT AGG ACG CCC GTT TCC ATT GAA    740
Arg Gln Ile Thr Asn Arg Tyr Thr Asp Arg Thr Pro Val Ser Ile Glu
        115                 120                 125

GAG ATC ACG GAT CTA ATC GAC GGC AAA GGA AGA TGC TCA TCT AAA GCA    788
Glu Ile Thr Asp Leu Ile Asp Gly Lys Gly Arg Cys Ser Ser Lys Ala
    130                 135                 140

AGA TAC CTT AGA AAC AAT GTA TAT GTT GAA GCG TTT GAC AGG GAT GCG    836
Arg Tyr Leu Arg Asn Asn Val Tyr Val Glu Ala Phe Asp Arg Asp Ala
145                 150                 155                 160

GGA GAA AAA CAA GTA CTT CTA AAA CCA TCA AAA TTC AAC ACG CCC GAA    884
Gly Glu Lys Gln Val Leu Leu Lys Pro Ser Lys Phe Asn Thr Pro Glu
                165                 170                 175

TCT AGG GCA TGG CAC ACG ACT AAT GAG ACG TAT ACC GTG TGG GGA TCA    932
Ser Arg Ala Trp His Thr Thr Asn Glu Thr Tyr Thr Val Trp Gly Ser
            180                 185                 190

CCA TGG ATA TAT CGA ACG GGA ACC TCC GTC AAT TGT ATA GTA GAG GAA    980
Pro Trp Ile Tyr Arg Thr Gly Thr Ser Val Asn Cys Ile Val Glu Glu
        195                 200                 205

ATG GAT GCC CGC TCT GTG TTT CCG TAT TCA TAT TTT GCA ATG GCC AAT   1028
Met Asp Ala Arg Ser Val Phe Pro Tyr Ser Tyr Phe Ala Met Ala Asn
    210                 215                 220

GGC GAC ATC GCG AAC ATA TCT CCA TTT TAT GGT CTA TCC CCA CCA GAG   1076
Gly Asp Ile Ala Asn Ile Ser Pro Phe Tyr Gly Leu Ser Pro Pro Glu
225                 230                 235                 240

GCT GCC GCA GAA CCC ATG GGA TAT CCC CAG GAT AAT TTC AAA CAA CTA   1124
Ala Ala Ala Glu Pro Met Gly Tyr Pro Gln Asp Asn Phe Lys Gln Leu
                245                 250                 255

GAT AGC TAT T‡‡ TCA ATG GAT TTG GAC AAG CGT CGA AAA GCA AGC CTT   1172
Asp Ser Tyr Phe Ser Met Asp Leu Asp Lys Arg Arg Lys Ala Ser Leu
            260                 265                 270

CCA GTC AAG CGT AAC T‡‡ CTC ATC ACA TCA CAC TTC ACA GTT GGG TGG   1220
Pro Val Lys Arg Asn Phe Leu Ile Thr Ser His Phe Thr Val Gly Trp
        275                 280                 285
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GAC TGG GCT CCA AAA ACT ACT CGT GTA TGT TCA ATG ACT AAG TGG AAA   1268
Asp Trp Ala Pro Lys Thr Thr Arg Val Cys Ser Met Thr Lys Trp Lys
    290                 295                 300

GAG GTG ACT GAA ATG TTG CGT GCA ACA GTT AAT GGG AGA TAC AGA TTT   1316
Glu Val Thr Glu Met Leu Arg Ala Thr Val Asn Gly Arg Tyr Arg Phe
305                 310                 315                 320

ATG GCC CGT GAA CTT TCG GCA ACG TTT ATC AGT AAT ACG ACT GAG TTT   1364
Met Ala Arg Glu Leu Ser Ala Thr Phe Ile Ser Asn Thr Thr Glu Phe
                325                 330                 335

GAT CCA AAT CGC ATC ATA TTA GGA CAA TGT ATT AAA CGC GAG GCA GAA   1412
Asp Pro Asn Arg Ile Ile Leu Gly Gln Cys Ile Lys Arg Glu Ala Glu
            340                 345                 350

GCA GCA ATC GAG CAG ATA TTT AGG ACA AAA TAT AAT GAC AGT CAC GTC   1460
Ala Ala Ile Glu Gln Ile Phe Arg Thr Lys Tyr Asn Asp Ser His Val
        355                 360                 365

AAG GTT GGA CAT GTA cAA TAT TTC TTG GCT CTC GGG GGA TTT ATT GTA   1508
Lys Val Gly His Val Gln Tyr Phe Leu Ala Leu Gly Gly Phe Ile Val
    370                 375                 380

GCA TAT CAG CCT GTT CTA TCC AAA TCC CTG GCT CAT ATG TAC CTC AGA   1556
Ala Tyr Gln Pro Val Leu Ser Lys Ser Leu Ala His Met Tyr Leu Arg
385                 390                 395                 400

GAA TTG ATG AGA GAC AAC AGG ACC GAT GAG ATG CTC GAC CTG GTA AAC   1604
Glu Leu Met Arg Asp Asn Arg Thr Asp Glu Met Leu Asp Leu Val Asn
                405                 410                 415

AAT AAG CAT GCA ATT TAT AAG AAA AAT GCT ACC TCA TTG TCA CGA TTG   1652
Asn Lys His Ala Ile Tyr Lys Lys Asn Ala Thr Ser Leu Ser Arg Leu
            420                 425                 430

CGG CGA GAT ATT CGA AAT GCA CCA AAT AGA AAA ATA ACA TTA GAC GAC   1700
Arg Arg Asp Ile Arg Asn Ala Pro Asn Arg Lys Ile Thr Leu Asp Asp
        435                 440                 445

ACC ACA GCT ATT AAA TCG ACA TCG TCT GTT CAA TTC GCC ATG CTC CAA   1748
Thr Thr Ala Ile Lys Ser Thr Ser Ser Val Gln Phe Ala Met Leu Gln
    450                 455                 460

TTT CTT TAT GAT CAT ATA CAA ACC CAT ATT AAT GAT ATG TTT AGT AGG   1796
Phe Leu Tyr Asp His Ile Gln Thr His Ile Asn Asp Met Phe Ser Arg
465                 470                 475                 480

ATT GCC ACA GCT TGG TGC GAA TTG CAG AAT AGA GAA CTT GTT TTA TGG   1844
Ile Ala Thr Ala Trp Cys Glu Leu Gln Asn Arg Glu Leu Val Leu Trp
                485                 490                 495
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CAC GAA GGG ATA AAG ATT AAT CCT AGC GCT ACA GCG AGT GCA ACA TTA 1892
His Glu Gly Ile Lys Ile Asn Pro Ser Ala Thr Ala ser Ala Thr Leu
500 505 510

GGA AGG AGA GTG GCT GCA AAG ATG TTG GGG GAT GTC GCT GCT GTA TCG 1940
Gly Arg Arg Val Ala Ala Lys Met Leu Gly Asp Val Ala Ala Val Ser
515 520 525

AGC TGC ACT GCT ATA GAT GCG GAA TCC GTC ACT TTG CAA AAT TCT ATG 1988
Ser Cys Thr Ala Ile Asp Ala Glu Ser Val Thr Leu Gln Asn Ser Met
530 535 540

CGA GTT ATC ACA TCC ACT AAT ACA TGT TAT AGC CGA CCA TTG GTT CTA 2036
Arg Val Ile Thr Ser Thr Asn Thr Cys Tyr Ser Arg Pro Leu Val Leu
545 550 555 560

TTT TCA TAT GGA GAA AAC CAA GGA AAC ATA CAG GGA CAA CTC GGT GAA 2084
Phe Ser Tyr Gly Glu Asn Gln Gly Asn Ile Gln Gly Gln Leu Gly Glu
565 570 575

AAC AAC GAG TTG CTT CCA ACG CTA GAG GCT GTA GAG CCA TGC TCG GCT 2132
Asn Asn Glu Leu Leu Pro Thr Leu Glu Ala Val Glu Pro Cys Ser Ala
580 585 590

AAT CAT CGT AGA TAT TTT CTG TTT GGA TCC GGT TAT GCT TTA TTT GAA 2180
Asn His Arg Arg Tyr Phe Leu Phe Gly Ser Gly Tyr Ala Leu Phe Glu
595 600 605

AAC TAT AAT TTT GTT AAG ATG GTA GAC GCT GCC GAT ATA CAG ATT GCT 2228
Asn Tyr Asn Phe Val Lys Met Val Asp Ala Ala Asp Ile Gln Ile Ala
610 615 620

AGC ACA TTT GTC GAG CTT AAT CTA ACC CTG CTA GAA GAT CGG GAA ATT 2276
Ser Thr Phe Val Glu Leu Asn Leu Thr Leu Leu Glu Asp Arg Glu Ile
625 630 635 640

TTG CCT TTA TCC GTT TAC ACA AAA GAA GAG TTG CGT GAT GTT GGT GTA 2324
Leu Pro Leu Ser Val Tyr Thr Lys Glu Glu Leu Arg Asp Val Gly Val
645 650 655

TTG GAT TAT GCA GAA GTA GCT CGC CGC AAT CAA CTA CAT GAA CTT AAA 2372
Leu Asp Tyr Ala Glu Val Ala Arg Arg Asn Gln Leu His Glu Leu Lys
660 665 670

TTT TAT GAC ATA AAC AAA GTA ATA GAA GTG GAT ACA AAT TAC GCG TTT 2420
Phe Tyr Asp Ile Asn Lys Val Ile Glu Val Asp Thr Asn Tyr Ala Phe
675 680 685

ATG AAC GGT TTG GCC GAA TTG TTT AAC GGT ATG GGT CAG GTA GGG CAA 2468
Met Asn Gly Leu Ala Glu Leu Phe Asn Gly Met Gly Gln Val Gly Gln
690 695 700

GCT ATA GGC AAA GTT GTA GTA GGG GCT GCC GGT GCA ATC GTA TCT ACC 2516
Ala Ile Gly Lys Val Val Val Gly Ala Ala Gly Ala Ile Val Ser Thr
705 710 715 720

ATA TCT GGT GTC TCT GCT TTC ATG TCA AAT CCC TTT GGG GCT TTG GCA 2564
Ile Ser Gly Val Ser Ala Phe Met Ser Asn Pro Phe Gly Ala Leu Ala
725 730 735

ATC GGT TTA ATC ATT ATA GCA GGA CTC GTG GCT GCA TTT TTA GCA TAT 26i2
Ile Gly Leu Ile Ile Ile Ala Gly Leu Val Ala Ala Phe Leu Ala Tyr
740 745 750

CGT TAT GTA AAC AAG CTT AAA AGC AAT CCA ATG AAA GCC CTT TAT CCT 2660
Arg Tyr Val Asn Lys Leu Lys Ser Asn Pro Met Lys Ala Leu Tyr Pro
755 760 765

ATG ACA ACA GAA GTG CTT AAG GCA CAG GCA ACG CGT GAG TTG CAT GGC 2708
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770 775 780

GAG GAA TCA GAT GAT TTG GAA CGA ACA TCT ATT GAT GAA AGA AAA TTA 2756
Glu Glu Ser Asp Asp Leu Glu Arg Thr Ser Ile Asp Glu Arg Lys Leu
785 790 795 800

GAA GAA GCT AGA GAA ATG ATA AAA TAT ATG GCG TTA GTC TCC GCG GAA 2804
Glu Glu Ala Arg Glu Met Ile Lys Tyr Met Ala Leu Val Ser Ala Glu
805 810 815

GAA CGC CAC GAG AAA AAA CTG CGG AGA AAG AGG CGA GGC ACT ACC GCC 2852
Glu Arg His Glu Lys Lys Leu Arg Arg Lys Arg Arg Gly Thr Thr Ala
820 825 830

GTT CTA TCG GAC CAC CTG GCA AAA ATG AGG ATT AAA AAT AGT AAC CCT 2900
Val Leu Ser Asp His Leu Ala Lys Met Arg Ile Lys Asn Ser Asn Pro
835 840 845

AAA TAT GAT AAG TTA CCT ACT ACA TAT TCA GAC TCA GAA GAT GAT GCT 2948
Lys Tyr Asp Lys Leu Pro Thr Thr Tyr Ser Asp Ser Glu Asp Asp Ala
850 855 860

GTG TAAGTGGGCA CTATTATATT TGAACTGAAT AAAACGCATA GAGCATGATA 3001
Val
865

TGGTTTACTC ATTTATTGCG AGATATAAAG CATATTCAAT ACGATATATT GCGAACGTGA 3061

TGCTAAAAAC ATAGCTCCCT GTATTATTGA TGCGCCATCA TTTGATTAAT AAATACATCG 3121

ACGCCGGCAT CACTGGTGCG GTGTATACCA GCTACGGCGC TAGCATTCAT GGTATCCCGT 3181

GATTGCTCGA TGCTTTCCTT CTGAATTC 3209

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 865 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

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Met His Tyr Phe Arg Arg Asn Cys Ile Phe Phe Leu Ile Val Ile Leu
 1               5                  10                  15

Tyr Gly Thr Asn Ser Ser Pro Ser Thr Gln Asn Val Thr Ser Arg Glu
            20                  25                  30

Val Val Ser Ser Val Gln Leu Ser Glu Glu Glu Ser Thr Phe Tyr Leu
        35                  40                  45

Cys Pro Pro Pro Val Gly Ser Thr Val Ile Arg Leu Glu Pro Pro Arg
    50                  55                  60

Lys Cys Pro Glu Pro Arg Lys Ala Thr Glu Trp Gly Glu Gly Ile Ala
65                  70                  75                  80

Ile Leu Phe Lys Glu Asn Ile Ser Pro Tyr Lys Phe Lys Val Thr Leu
                85                  90                  95

Tyr Tyr Lys Asn Ile Ile Gln Thr Thr Thr Trp Thr Gly Thr Thr Tyr
            100                 105                 110

Arg Gln Ile Thr Asn Arg Tyr Thr Asp Arg Thr Pro Val Ser Ile Glu
        115                 120                 125

Glu Ile Thr Asp Leu Ile Asp Gly Lys Gly Arg Cys Ser Ser Lys Ala
    130                 135                 140

Arg Tyr Leu Arg Asn Asn Val Tyr Val Glu Ala Phe Asp Arg Asp Ala
145                 150                 155                 160

Gly Glu Lys Gln Val Leu Leu Lys Pro Ser Lys Phe Asn Thr Pro Glu
                165                 170                 175

Ser Arg Ala Trp His Thr Thr Asn Glu Thr Tyr Thr Val Trp Gly Ser
            180                 185                 190

Pro Trp Ile Tyr Arg Thr Gly Thr Ser Val Asn Cys Ile Val Glu Glu
        195                 200                 205

Met Asp Ala Arg Ser Val Phe Pro Tyr Ser Tyr Phe Ala Met Ala Asn
    210                 215                 220
```

Gly Asp Ile Ala Asn Ile Ser Pro Phe Tyr Gly Leu Ser Pro Pro Glu
225 230 235 240

Ala Ala Ala Glu Pro Met Gly Tyr Pro Gln Asp Asn Phe Lys Gln Leu
245 250 255

Asp Ser Tyr Phe Ser Met Asp Leu Asp Lys Arg Arg Lys Ala Ser Leu
260 265 270

Pro Val Lys Arg Asn Phe Leu Ile Thr Ser His Phe Thr Val Gly Trp
275 280 285

Asp Trp Ala Pro Lys Thr Thr Arg Val Cys Ser Met Thr Lys Trp Lys
290 295 300

Glu Val Thr Glu Met Leu Arg Ala Thr Val Asn Gly Arg Tyr Arg Phe
305 310 315 320

Met Ala Arg Glu Leu Ser Ala Thr Phe Ile Ser Asn Thr Thr Glu Phe
325 330 335

Asp Pro Asn Arg Ile Ile Leu Gly Gln Cys Ile Lys Arg Glu Ala Glu
340 345 350

Ala Ala Ile Glu Gln Ile Phe Arg Thr Lys Tyr Asn Asp Ser His Val
355 360 365

Lys Val Gly His Val Gln Tyr Phe Leu Ala Leu Gly Gly Phe Ile Val
370 375 380

Ala Tyr Gln Pro Val Leu Ser Lys Ser Leu Ala His Met Tyr Leu Arg
385 390 395 400

Glu Leu Met Arg Asp Asn Arg Thr Asp Glu Met Leu Asp Leu Val Asn
405 410 415

Asn Lys His Ala Ile Tyr Lys Lys Asn Ala Thr Ser Leu Ser Arg Leu
420 425 430

Arg Arg Asp Ile Arg Asn Ala Pro Asn Arg Lys Ile Thr Leu Asp Asp
435 440 445

Thr Thr Ala Ile Lys Ser Thr Ser Ser Val Gln Phe Ala Met Leu Gln
450 455 460

Phe Leu Tyr Asp His Ile Gln Thr His Ile Asn Asp Met Phe Ser Arg
465 470 475 480

Ile Ala Thr Ala Trp Cys Glu Leu Gln Asn Arg Glu Leu Val Leu Trp
485 490 495

His Glu Gly Ile Lys Ile Asn Pro Ser Ala Thr Ala Ser Ala Thr Leu
500 505 510

Gly Arg Arg Val Ala Ala Lys Met Leu Gly Asp Val Ala Ala Val Ser
515 520 525

Ser Cys Thr Ala Ile Asp Ala Glu Ser Val Thr Leu Gln Asn Ser Met
530 535 540

Arg Val Ile Thr Ser Thr Asn Thr Cys Tyr Ser Arg Pro Leu Val Leu
545 550 555 560

Phe Ser Tyr Gly Glu Asn Gln Gly Asn Ile Gln Gly Gln Leu Gly Glu
565 570 575

Asn Asn Glu Leu Leu Pro Thr Leu Glu Ala Val Glu Pro Cys Ser Ala
580 585 590

Asn His Arg Arg Tyr Phe Leu Phe Gly Ser Gly Tyr Ala Leu Phe Glu
595 600 605

Asn Tyr Asn Phe Val Lys Met Val Asp Ala Ala Asp Ile Gln Ile Ala
610 615 620

Ser Thr Phe Val Glu Leu Asn Leu Thr Leu Leu Glu Asp Arg Glu Ile
625 630 635 640

Leu Pro Leu Ser Val Tyr Thr Lys Glu Glu Leu Arg Asp Val Gly Val
645 650 655

Leu Asp Tyr Ala Glu Val Ala Arg Arg Asn Gln Leu His Glu Leu Lys
660 665 670

Phe Tyr Asp Ile Asn Lys Val Ile Glu Val Asp Thr Asn Tyr Ala Phe
675 680 685

Met Asn Gly Leu Ala Glu Leu Phe Asn Gly Met Gly Gln Val Gly Gln
690 695 700

Ala Ile Gly Lys Val Val Val Gly Ala Ala Gly Ala Ile Val Ser Thr
705 710 715 720

Ile Ser Gly Val Ser Ala Phe Met Ser Asn Pro Phe Gly Ala Leu Ala
725 730 735

Ile Gly Leu Ile Ile Ile Ala Gly Leu Val Ala Ala Phe Leu Ala Tyr
740 745 750

Arg Tyr Val Asn Lys Leu Lys Ser Asn Pro Met Lys Ala Leu Tyr Pro
755 760 765

Met Thr Thr Glu Val Leu Lys Ala Gln Ala Thr Arg Glu Leu His Gly
770 775 780

Glu Glu Ser Asp Asp Leu Glu Arg Thr Ser Ile Asp Glu Arg Lys Leu
785 790 795 800

Glu Glu Ala Arg Glu Met Ile Lys Tyr Met Ala Leu Val Ser Ala Glu
805 810 815

Glu Arg His Glu Lys Lys Leu Arg Arg Lys Arg Arg Gly Thr Thr Ala
820 825 830

Val Leu Ser Asp His Leu Ala Lys Met Arg Ile Lys Asn Ser Asn Pro
835 840 845

Lys Tyr Asp Lys Leu Pro Thr Thr Tyr Ser Asp Ser Glu Asp Asp Ala
850 855 860

Val
865

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CGAATTCGTC GAC 13

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AATTGTCGAC GAATTCGAGC T 21

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 34 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

ACTCAATCAA TAGCAATCAT GCACTATTTT AGGC 34

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICs:

(A) LENGTH: 26 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCGGAATTGC ATATTTTTCC TTATAG 26

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GGATCCAAT CATGCACTAT TTTAGG 26

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CCATATATAT TCCCTACTAT TCCCCGCGGC GGTTCTAGAC 40

Claims

1. A recombinant fowlpox virus comprising a gene coding for an antigen of Marek's Disease Virus under control of a poxvirus promoter within a region of the DNA of fowlpox virus which is not essential for virus growth.

2. The recombinant fowlpox virus of claim 1, wherein said antigen gene is a gene from Marek's disease virus encoding a protein selected from the group consisting of glycoprotein B homologue, glycoprotein C homologue, glycoprotein D homologue, glycoprotein H homologue and tegument proteins.

3. The recombinant fowlpox virus of Claim 1, wherein the promoter-antigen gene is inserted with lacZ gene of *E. coli* under the control of another poxvirus promoter.

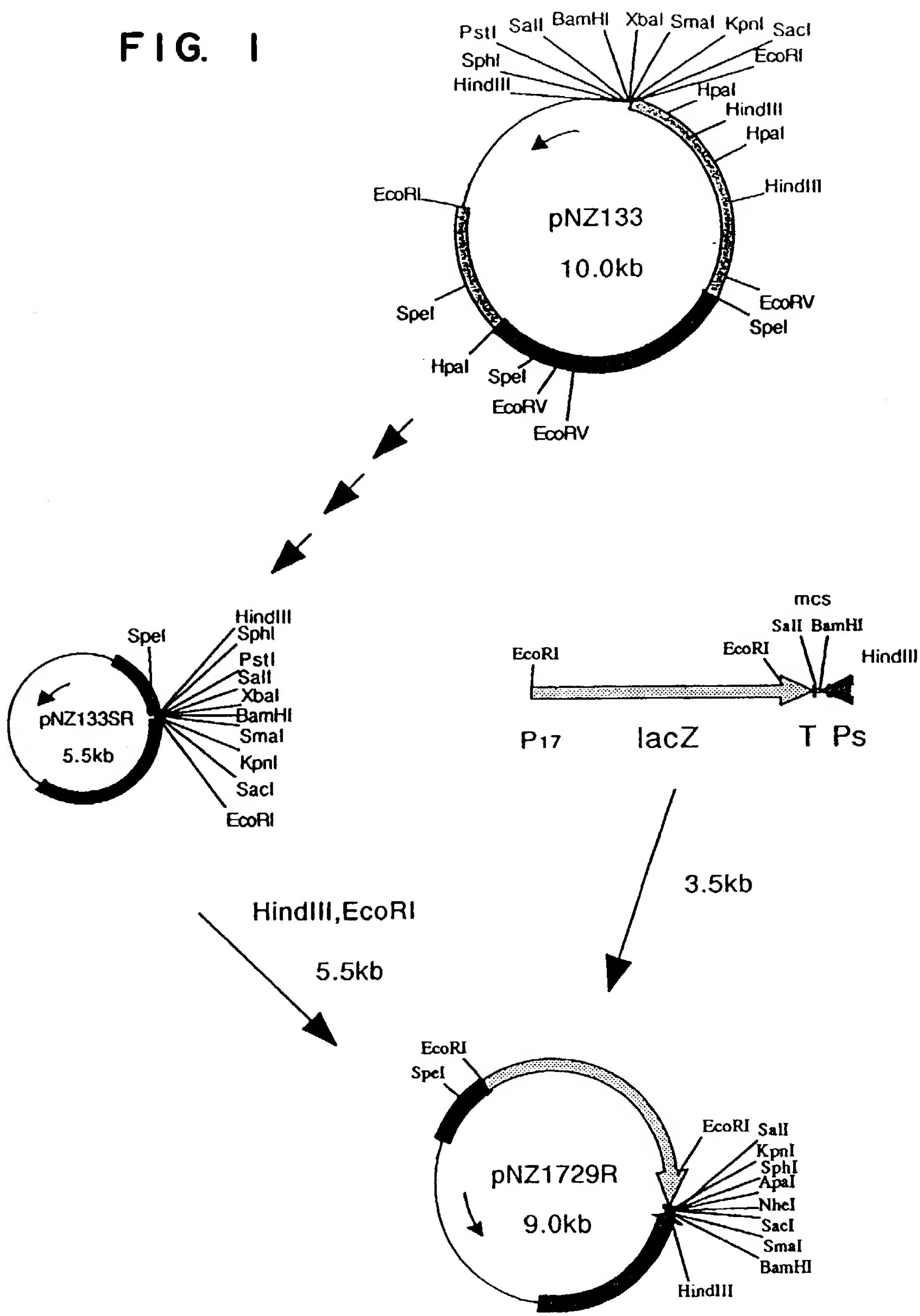
4. The recombinant FPV of Claim 1 or wherein said antigen gene is glycoprotein B homologue of Marek's Disease Virus.

5. A vaccine composition comprising:
   an effective amount of the recombinant fowlpox virus of claim 1; and
   a pharmaceutically acceptable carrier.

6. The vaccine composition of claim 5, in a cell-free lyophilized state.

7. The vaccine composition of claim 5, in a cell-free frozen state.

FIG. 1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HpaI
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EcoRV
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XbaI
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SmaI
KpnI
SacI
EcoRI
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EcoRI
SalI
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ApaI
NheI
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HindIII
pNZ1729R
9.0kb

FIG. 2

```
SEQ.
ID. NO.

                         FPV promoter            start
 1   5'-AATTCGAGCTCGGATCGTTGAAAAAATAATATAGATCCTAAAATGGAA      -3'
 2   3'-      GCTCGAGCCTAGCAACTTTTTTATTATATCTAGGATTTTACCTTCTAG-5'
       EcoRI

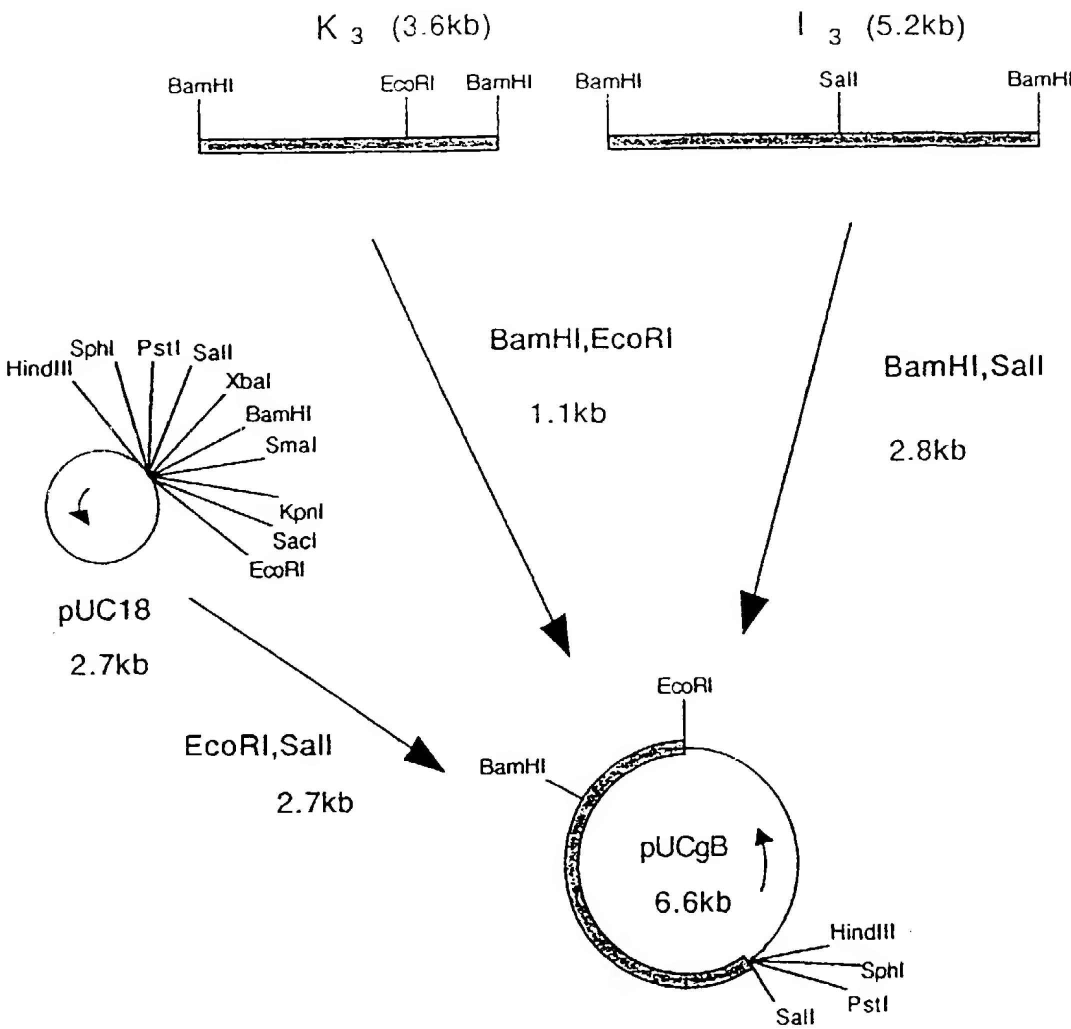
 3   5'-AGCTTTTTTTTTTTTTTTTTTTTGGCATATAAATAATAAATACAATAATTAATTA      -3'
 4   3'-      AAAAAAAAAAAAAAAAAAACCGTATATTTATTATTTATGTTATTAATTAATGCGC-5'
       HindIII                                                  MluI

 5   5'-CGCGTAAAAATTGAAAAACTATTCTAATTTATTGCACTCG     -3'
 6   3'-      ATTTTTAACTTTTTGATAAGATTAAATAACGTGAGCCTAG-5'
       MluI                                        BamHI

 7   5'-GATCCCCGGGCGAGCTCGCTAGCGGGCCCGCATGCGGTACCG     -3'
 8   3'-      GGGCCCGCTCGAGCGATCGCCCGGGCGTACGCCTAGGCAGCT-5'
       BamHI SmaI   SacI  NheI  ApaI  SphI  KpnI SalI

           both directional terminator
 9   5'-TCGACCCGGTACATTTTTATAAAAATGTACCCGGGGATC-3'
10   3'-      GGGCCATGTAAAAATATTTTTACATGGGCCCCTAG-5'
```

FIG. 3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I 3 (5.2kb)
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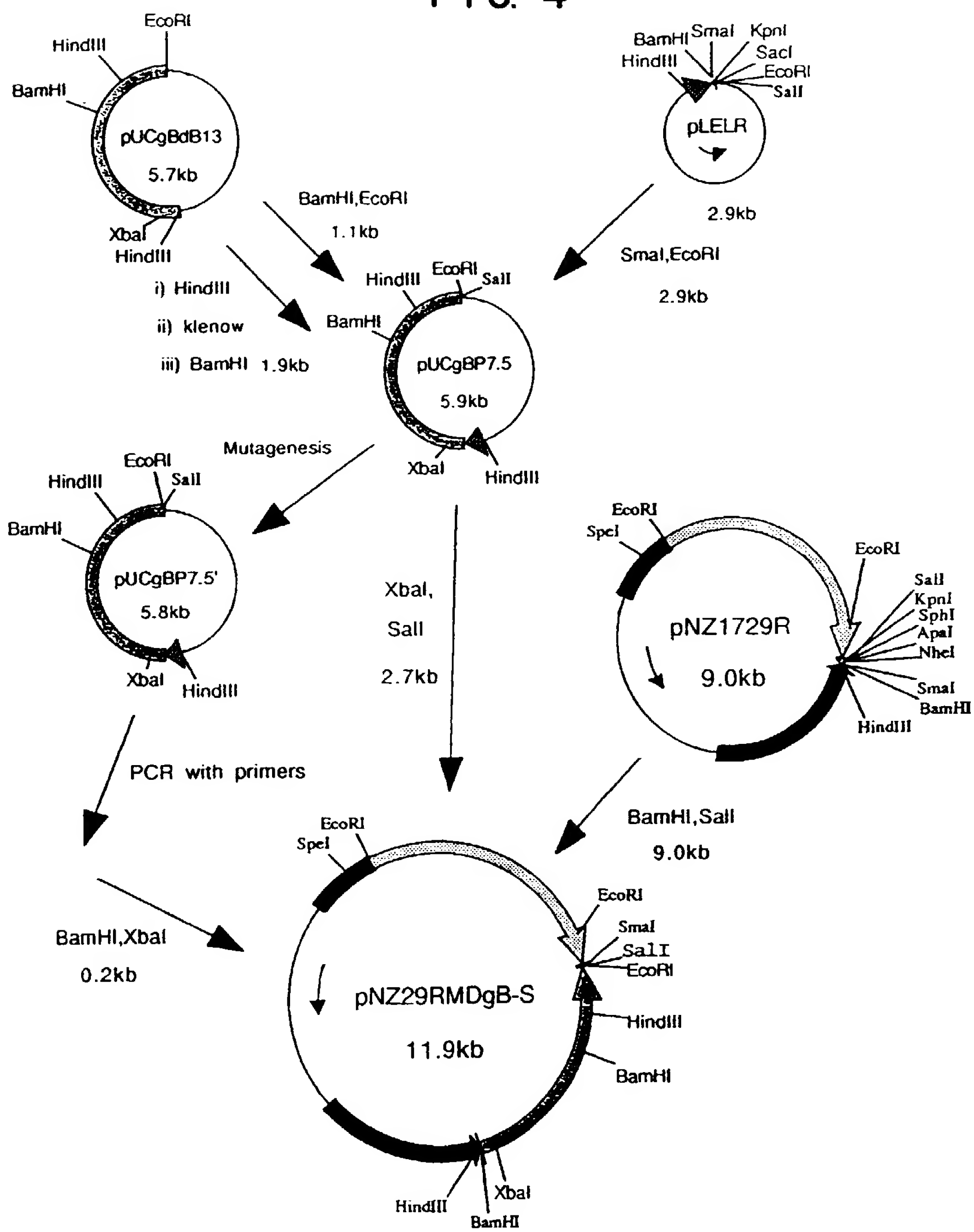
SalI

FIG. 4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SacI
HindIII
EcoRI
SalI
pLELR
2.9kb
BamHI,EcoRI
1.1kb
i) HindIII
ii) klenow
iii) BamHI 1.9kb
SmaI,EcoRI
2.9kb
HindIII
EcoRI
SalI
BamHI
pUCgBP7.5
5.9kb
XbaI
HindIII
Mutagenesis
EcoRI
HindIII
SalI
BamHI
pUCgBP7.5'
5.8kb
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HindIII
XbaI,
SalI
2.7kb
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SpeI
EcoRI
SalI
KpnI
SphI
ApaI
NheI
pNZ1729R
9.0kb
SmaI
BamHI
HindIII
PCR with primers
BamHI,SalI
9.0kb
EcoRI
SpeI
EcoRI
SmaI
SalI
EcoRI
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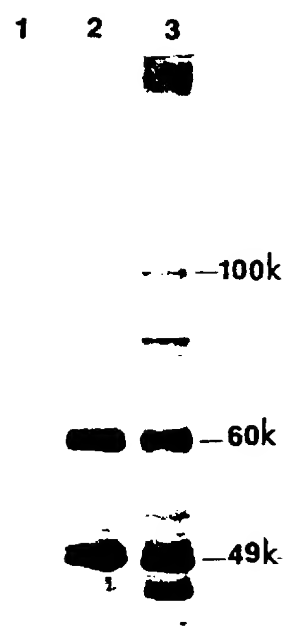
HindIII
BamHI
HindIII
XbaI
BamHI

FIG. 5



European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 92 30 5775
Page 1

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
P,X	JOURNAL OF VIROLOGY vol. 66, no. 3, March 1992, pages 1402 - 1408 YANAGIDA, N. ET AL. 'Recombinant Fowlpox viruses expressing the glycoprotein B homolog and the pp38 gene of Marek's disease virus' * the whole document * ---	1-4	C12N15/38 C12N15/86 A61K39/255
P,X	JOURNAL OF VIROLOGY vol. 66, no. 3, March 1992, pages 1409 - 1413 NAZERIAN,K. ET AL. 'Protection against Marek's disease by a Fowlpox virus recombinant expressing the glycoprotein B of Marek's disease virus' * the whole document * ---	1-7	
X,D	EP-A-0 284 416 (NIPPON ZEON) * page 4, line 48 *	1,3,5	
Y	* the whole document * ---	1-5	TECHNICAL FIELDS SEARCHED (Int. Cl.5)
X	EP-A-0 314 569 (TRANSGENE) * the whole document *	1,3,5	C07K C12N A61K
Y	* the whole document * ---	1-5	
X	WO-A-8 912 684 (NATIONAL RESEARCH DEVELOPMENT CORPORATION) * page 2, line 31; figure 6; example 1 *	1,3,5	
Y	* the whole document * ---	1-5	
D,Y	WO-A-9 002 803 (RHONE-MERIEUX) * the whole document * --- -/--	1-5	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	19 OCTOBER 1992	CHAMBONNET F.J.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
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European Patent Office

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Application Number

EP 92 30 5775
Page 2

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
D,Y	JOURNAL OF GENERAL VIROLOGY vol. 70, 1989, READING, UK pages 1789 - 1804 ROSS, L. J. N. 'Nucleotide sequence and characterization of the Marek's Disease virus homologue of glycoprotein B of Herpes Simplex Virus' * the whole document *	1-5	
D,Y	JOURNAL OF GENERAL VIROLOGY vol. 72, no. 4, April 1991, READING UK pages 939 - 947 ROSS, L.J.N. & BINNS, M.M. 'Properties and evo.utionary relationships of the Marek's disease virus homologues of protein kinase, glycoprotein d and glycoprotein I of herpes simplex virus' * the whole document *	1-5	

TECHNICAL FIELDS SEARCHED (Int. Cl.5)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	19 OCTOBER 1992	CHAMBONNET F.J.

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L : document cited for other reasons

& : member of the same patent family, corresponding document

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